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In Vitro Processing of Human Immunodeficiency Virus Type 1 Gag Virus-like Particles

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Human immunodeficiency virus (HIV) Gag proteins are assembled into virus particles and then cleaved by the virion-associated HIV protease. Concomitant with Gag processing, doughnut-like HIV particles (the immature form) are converted to particles containing condensed cores (the mature form). Here we describe the *in vitro* processing of immature HIV Gag virus-like particles (VLP) by exogenously added HIV protease. Following delipidization, sequential processing of immature VLP showed that the matrix (MA)/capsid (CA) junction was cleaved faster than the CA/nucleocapsid (NC) junction, an altered order of processing when compared with authentic processing. When the *in vitro* processed VLP were analyzed on density gradients, most of the MA, CA-p15 intermediate, and NC were detected as a highly multimeric form, equivalent to the unprocessed VLP. In contrast, CA was found as a monomer dissociated from the multimeric CA-p15 following cleavage of the CA/NC junction. Electron microscopy revealed that the *in vitro* processing was accompanied by conversion of the doughnut-like particles to particles containing condensed cores and spherical outer shells. The cores, however, lacked core shells, which are normally observed for authentic HIV, suggesting that the *in vitro* processing of immature VLP failed to produce core shells.

INTRODUCTION

The Gag protein is the main structural component of retroviral particles and also drives virus particle budding. The Gag protein of human immunodeficiency virus (HIV) is synthesized initially as a precursor polyprotein that self-assembles underneath the plasma membrane to form the budding virus particle. Just after budding the particle is often seen as a hollow sphere surrounded by an electron-dense double ring structure that is composed of multimerized Gag precursors, the immature form. However, during or after budding (Kaplan *et al.,* 1994; Vogt, 1996), Gag polyprotein undergoes the process termed maturation in which it is cleaved by viral protease (PR) to yield the N-terminal matrix (MA), the central capsid (CA), the nucleocapsid (NC), and the C-terminal p6 proteins (Mervis *et al.,* 1988). Concomitant with Gag processing, the virus particle adopts a submembrane shell enclosing an electron-dense core surrounded by a core shell. From immunoelectron microscopic examination, it is widely accepted that the submembrane shell is composed of MA protein while the electron-dense core contains condensed NC protein with genomic RNA surrounded by the core shell composed of CA protein (Nermut and Hockley, 1996). Expression of Gag protein alone produces immature virus particles (Gheysen *et al.,* 1989; Hoshikawa *et al.,* 1991; Smith *et al.,* 1993), while that of Gag protein with a PR region results in the mature form of the virus particle (Hoshikawa *et al.,* 1991; Karacostas *et al.,* 1989). The obligatory role for PR in Gag processing and the accompanying morphological conversion have been demonstrated by studies in which expression of Gag with mutated inactive forms of PR fails to cleave Gag proteins and results in the production of virus particles arrested at the immature stage (Gottlinger *et al.,* 1989; Park and Morrow, 1993; Rose *et al.,* 1995).

Since immature retroviruses are noninfectious, the processing of HIV Gag has been studied extensively as a target for antiviral therapy. In HIV-infected cells and in expression systems using Gag recombinant viruses, the cleavage of the MA/CA, CA/p2, p2/NC, NC/p1, and p1/p6 junctions occurs at different rates, suggesting that each site is differentially susceptible to cleavage. When the cleavage sites are made uncleavable by mutation, particles with aberrant morphologies are produced, suggesting that correct cleavage is essential for virus maturation (Accola *et al.,* 1998; Gottlinger *et al.,* 1989; Krausslich *et al.,* 1995; Wiegers *et al.,* 1998). However, it is difficult to determine the order of Gag processing in cell-based experiments as the process of virus particle budding and that of Gag processing are interlinked and neither process is synchronized. To overcome this difficulty, *in vitro* translated Gag protein has been widely used for cleavage studies by exogenously added PR, and these studies have shown the production of authentic cleavage products, suggesting an order of processing (Krausslich *et*

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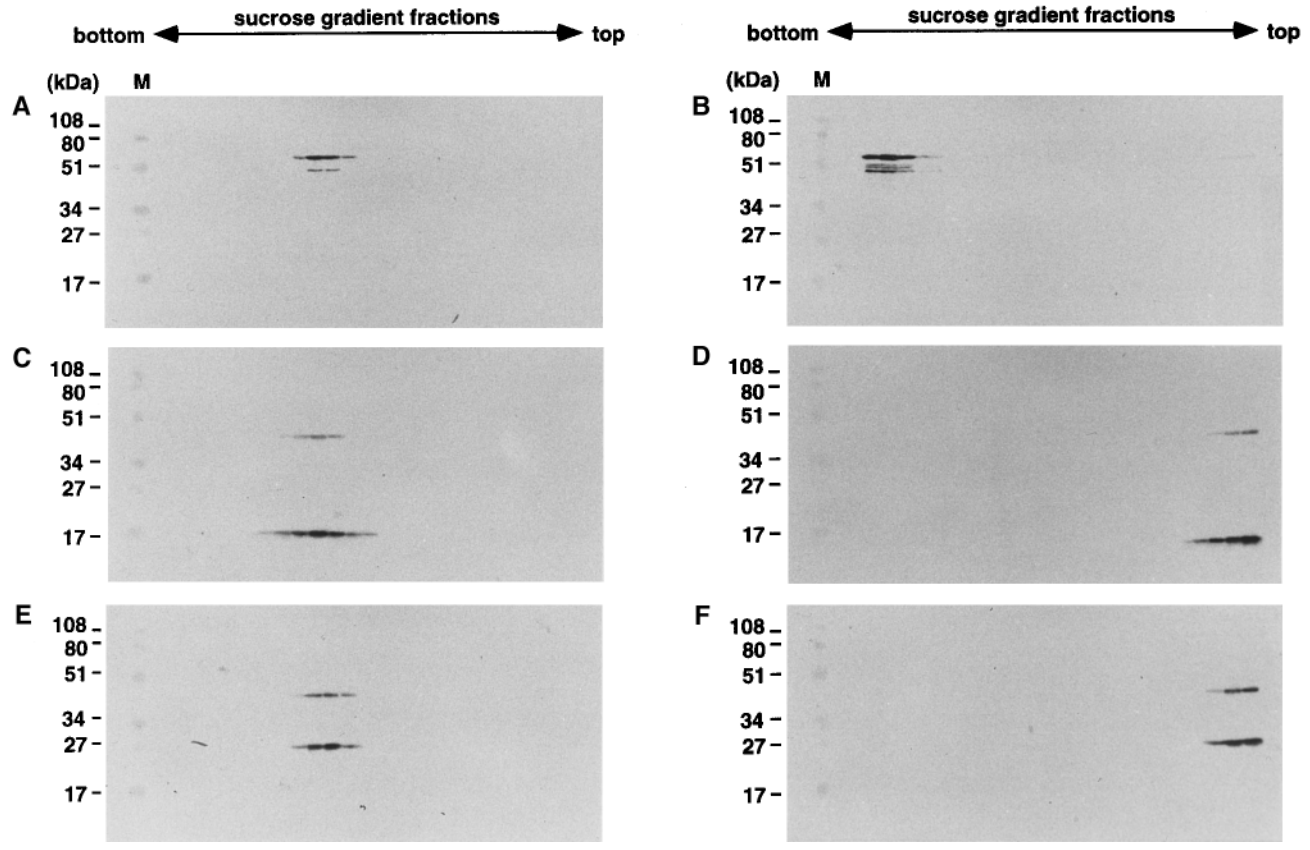

FIG. 1. Gradient analysis of immature and mature forms of VLPs treated with detergent. Purified VLP were adjusted to 0.5% Triton X-100 and held for 30 min before being layered onto 20–70% (w/v) sucrose density gradients. Centrifugation was carried out in an SW55 rotor at 4°C at 120,000 *g* for 4 h. Fractions were collected from the bottom to the top (left to right) and analyzed by SDS–PAGE followed by Western blotting using anti-HIV-1 MA (C and D) or CA (A, B, E, and F) monoclonal antibodies. (A and B) Immature VLP; (C–F) mature VLP. (Panels on the right-hand side) Treated with detergent; (panels on the left-hand side) untreated. Lane M, prestained molecular weight markers (Bio-Rad).

al., 1998, 1989; Pettit *et al.,* 1994; Tritch *et al.,* 1991). Although such systems use authentic substrates for cleavage rather than oligopeptides containing the cleavage site, the morphological conversion of the HIV particle that accompanies Gag processing cannot be followed. By using a purified source of immature HIV particles as a substrate, however, *in vitro* processing and direct observation of the accompanying morphological changes might be possible. Here we describe a system of *in vitro* processing of immature HIV Gag virus-like particles (VLP) by exogenously added PR and the cleavage and conversion processes observed.

RESULTS

Removal of the VLP lipid bilayer by detergent treatment

As Gag proteins enveloped by the lipid bilayer are resistant to protease digestion (Konvalinka *et al.,* 1995; Lee and Yu, 1998; Spearman and Ratner, 1996), we disrupted the lipid bilayer of VLP before assessing Gag processing *in vitro.* As Gag shells of immature retroviruses (e.g., avian leukosis virus and HIV) have been reported to be stable in nonionic detergents (Lee and Yu, 1998; Rose *et al.,* 1995; Stewart *et al.,* 1990), initial membrane permeabilization was carried out by treatment with 0.5% Triton X-100. When the delipidized Gag shell of immature HIV VLP was centrifuged on a 20–70% sucrose density gradient, it passed through the least dense gradient fractions and migrated further than the untreated VLP (Figs. 1A and 1B), indicating that unprocessed Gag shells were stable in the presence of detergent. The position of Gag shells in the gradient at a density of 1.22 g/ml compared to 1.17 g/ml for VLP is consistent with the observations that denuded immature HIV Gag shells are denser than immature HIV particles with the lipid bilayer intact (Lee and Yu, 1998; Lingappa *et al.,* 1997). In contrast, when the mature form of HIV VLP was treated with the detergent and similarly centrifuged, MA, CA, and the MA-CA intermediate (designated p39 in Gowda *et al.,* 1989; Pettit *et al.,* 1994) were detected in the least dense gradient fractions by Western blotting using anti-MA (Figs. 1C and 1D) and anti-CA (Figs. 1E and 1F) monoclonal antibodies, confirming previous reports that the interactions of processed Gag proteins are sensitive to detergent treatment (Lee and Yu, 1998; Stewart *et al.,* 1990).

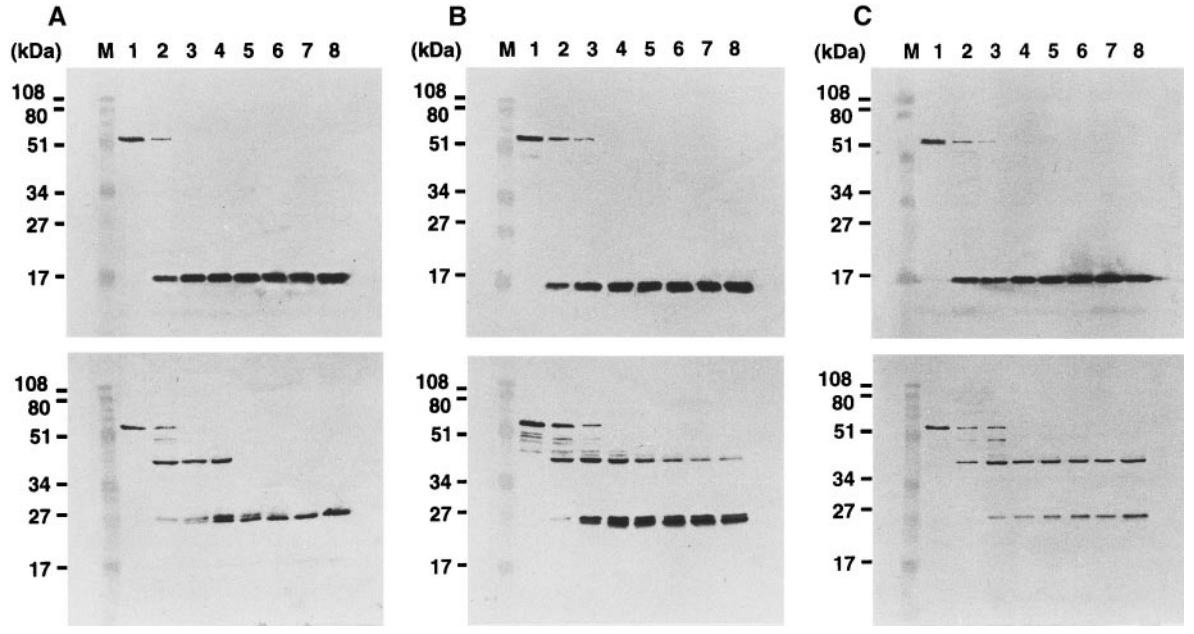

FIG. 2. Sequential processing of Gag shells by PR *in vitro* under different conditions. Immature VLP were treated with 0.5% Triton X-100 for 30 min, diluted with 10 vol of PBS, and centrifuged at 4°C at 150,000 *g* for 2 h. The delipidized Gag shells were resuspended in buffer containing 50 mM MES (pH 6.0), 150 mM NaCl, 1 mM DTT, and 1 mM EDTA (A), in buffer containing 50 mM MES (pH 6.0), 500 mM NaCl, 1 mM DTT, and 1 mM EDTA (B), or in buffer containing 50 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM DTT, and 1 mM EDTA (C). Digestion by purified HIV-1 PR was carried out at 37°C in 100 μl of buffer (described above) containing 35–40 μg of Gag and 250 ng of PR (Gag-to-PR molar ratio of 30:1). The digested products were detected by Western blotting using anti-HIV-1 MA (top) and CA (bottom). Lanes: M, prestained molecular weight markers (Bio-Rad); 1, prior to digestion; 2–8, digested for 3, 10, 30, 60, 120, 240, and 480 min, respectively.

In vitro processing of delipidized VLP

In vitro processing of VLP was performed with the immature VLP following treatment with 0.5% Triton X-100. Detergent was removed by dilution with an excess volume of PBS and the Gag shells were re-pelleted by centrifugation before incubation with recombinant HIV-1 PR. To confirm previous studies in which the optimum activity for HIV-1 PR was observed at mildly acidic pH and at low salt concentration (Konvalinka *et al.,* 1995), sequential processing was carried out in buffers of pH 6 or 7 with different concentrations of salt. The products of digestion were subjected to SDS–PAGE followed by Western blotting using anti-MA (Fig. 2, top) and anti-CA antibodies (Fig. 2, bottom). Under all conditions tested, the MA domain was promptly released (Fig. 2, top) while, in contrast, CA(p24) and CA-p2(p25) were more gradually released concomitant with the disappearance of the processing intermediate CA-p15 (designated p40 in Pettit *et al.,* 1994) that was identified both by the absence of reactivity with anti-MA antibody (Fig. 2, top) and by the reactivity with an anti-p6 peptide serum (data not shown). The reaction carried out at pH 6 in 150 mM salt was essentially complete by 2 h post-PR addition (Fig. 2A). However, the reaction was retarded by a high concentration of salt (Fig. 2B). Severe retardation of cleavage was observed at pH 7 and no mature CA(p24) was produced throughout the digestion period (Fig. 2C, bottom). Thus, as the digestion of Gag shells at neutral pH resulted in incomplete processing, all further study of *in vitro* processing was performed at pH 6 in 150 mM salt.

To investigate the multimeric states of the processed Gag proteins, the *in vitro* processing products were centrifuged on 20–70% sucrose density gradients and detected by Western blotting using anti-MA and anti-CA antibodies (Fig. 3). Most of the MA protein was found in the same fractions as the unprocessed Gag shells following removal of the lipid bilayer, indicating that MA molecules was retained in a highly multimeric form, although a trace of MA was found in the least dense fractions (Fig. 3A). When probed with anti-CA antibody, most of the processing intermediate was also observed as a highly multimeric form, as was a small fraction of the processed CA protein. Most of the CA protein, however, was found in the least dense fractions, suggesting that although some CA molecules existed in a highly multimeric form, the majority of the CA molecules were released as low-molecular-weight forms by PR digestion (Fig. 3B, top). The dissociated form of CA accumulated during the course of the reaction while, in contrast, the multimeric forms of the processing intermediates and those of the CA molecules were reduced (Fig. 3B, bottom). When the *in vitro* products were subjected to sedimentation analysis on a 15–30% glycerol gradient and compared with molecular mass markers sedimented in parallel (Fig. 4), the calculated molecular mass of the dissociated CA protein was 24–25 kDa, equivalent to the

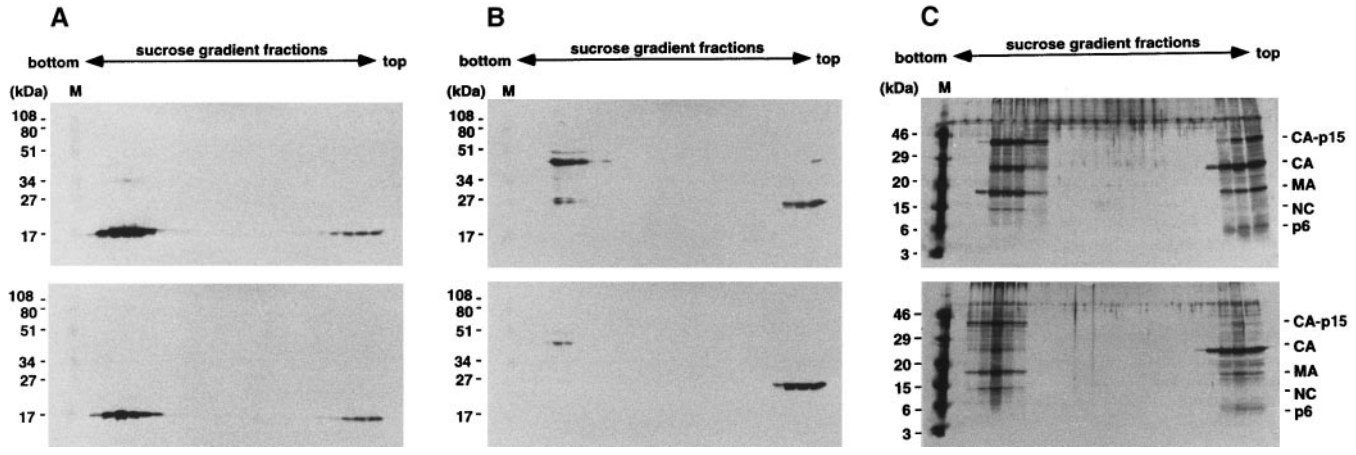

FIG. 3. Gradient analysis of *in vitro* processed Gag shells. Preparation of delipidized Gag shells was performed as described in the legend to Fig. 2. The Gag shells were resuspended in buffer containing 50 mM MES (pH 6.0), 150 mM NaCl, 1 mM DTT, and 1 mM EDTA and digested by HIV-1 PR at 37°C for 30 min (top) or 2 h (bottom) in 100-μl reaction mixtures containing 35–40 μg of Gag and 250 ng of PR (Gag-to-PR molar ratio of 30:1). The digested products were applied to 20–70% (w/v) sucrose gradients and centrifuged in an SW55 rotor at 4°C at 120,000 *g* for 4 h. Fractions from the bottom to the top (left to right) were analyzed by SDS–PAGE and then subjected either to Western blotting using anti-HIV-1 MA (A) or CA (B) monoclonal antibodies or to silver staining (C). Lane M, prestained molecular weight markers (Bio-Rad in A and B or Life Technologies in C).

monomeric form, not the dimeric form (Fig. 4C). These results suggest that (1) CA domains were released largely as monomers from highly multimeric processing intermediates after cleavage of the CA/NC junction and (2) the multimeric CA species were only transient forms of CA during the *in vitro* processing reaction. As an informative anti-NC antibody was not available, the sedimented position of the NC protein in a 20–70% sucrose gradient was detected directly by silver staining. The NC protein was found in the same fractions as the MA protein and the processing intermediate, suggesting that the NC protein also exists in a highly multimeric form (Fig. 3C). Visible in the lightest fractions of the gradient was a 6-kDa protein, probably the C-terminal p6 domain. When *in vitro* processing was carried out at pH 7 or at high salt concentration and similarly analyzed on 20–70% sucrose gradients, no significant differences were observed in the sedimentation profiles (data not shown).

To examine the possibility that the observed dissociation of CA protein during the processing reaction may be due to trace amounts of the detergent remaining in the Gag shell preparation, delipidization was also performed by extraction with ether and followed by similar digestion with PR. The pattern of sequential processing as identified by anti-MA and anti-CA antibodies was similar to that shown for detergent-extracted Gag (data not shown). When the *in vitro* processed Gag shell was sedimented through a 20–70% sucrose gradient, the profiles were found to be similar to those of the Gag shell digested by PR after the detergent treatment (Fig. 5). The MA protein (Fig. 5B), the processing intermediate (Fig. 5C), and the NC protein (Fig. 5D) were found as highly multimeric forms while, in contrast, the CA protein was present only in the lightest gradient fractions (Fig. 5C). These results indicate that the dissociation of CA from the Gag shell following *in vitro* processing is not due to the residual detergent in the shell preparation but rather to the lack of a stable interaction between the CA domains after PR processing.

Detergent sensitivity of *in vitro* processed VLP

As Gag–gag interactions of authentic mature virions are sensitive to 0.5% Triton X-100 (cf. Fig. 1), the effect of detergent addition to the *in vitro* processed VLP was examined. The highly multimeric forms of Gag following *in vitro* processing were recovered from the relevant gradient fractions and re-pelleted by centrifugation. Following the addition of 0.5% Triton X-100, the Gag preparation was reanalyzed on a 20–70% sucrose density gradient. Fractionation of the gradient showed that all the multimeric MA, processing intermediate, and CA were dissociated (Figs. 6A and 6B), similar to the disruption of mature VLP by the detergent treatment (compare to Figs. 1D and 1E, respectively). This finding suggests, although it does not prove, that multimeric Gag proteins processed *in vitro* and *in vivo* are structurally and organizationally similar. By contrast, when delipidized Gag shells were not digested by PR, further treatment with 0.5% Triton X-100 did not disrupt the highly multimeric state of Gag proteins (Fig. 6C).

Morphological conversion of VLP by *in vitro* processing

Electron microscopic analysis of the products of the *in vitro* digestion reaction was done to examine whether the material had a defined structure and/or similarity to mature HIV. The Gag shell delipidized by treatment with 0.5% Triton X-100 showed a hollow sphere surrounded by a double ring structure (Fig. 7B), typical of untreated

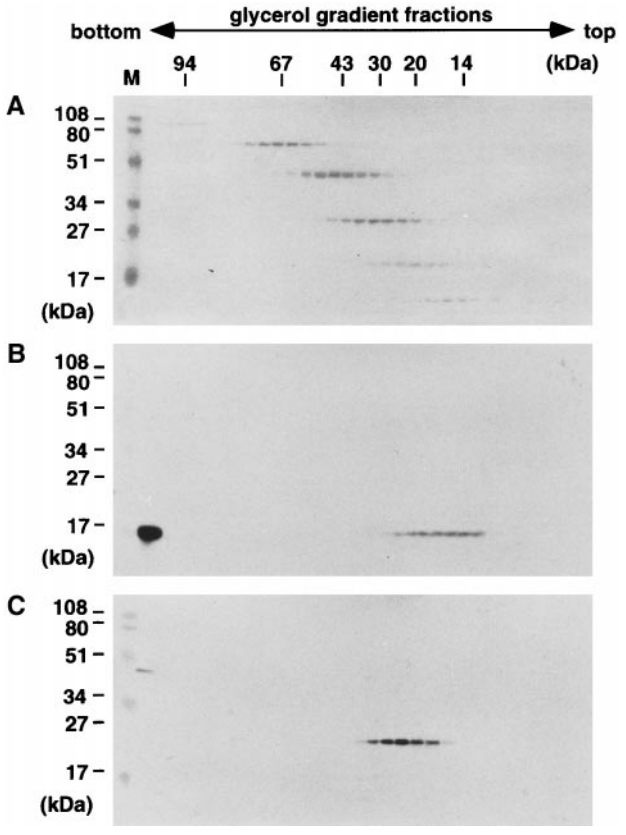

FIG. 4. Sedimentation analysis to determine the molecular masses of the dissociated mature Gag proteins following *in vitro* processing. Delipidization and digestion of Gag VLP were performed as described in the legends to Figs. 2 and 3, respectively. The 2-h digested products were subjected to velocity sedimentation analysis on 15–30% (v/v) glycerol gradients. Centrifugation was carried out in an SW55 rotor at 4°C at 230,000 *g* for 40 h. Fractions from the bottom to the top (left to right) were analyzed by SDS–PAGE followed by Western blotting using anti-HIV-1 MA (B) or CA (C) monoclonal antibodies. Molecular mass markers (a low-molecular-weight calibration kit, Amersham Pharmacia Biotech) were sedimented in parallel and stained with Coomassie brilliant blue (A). Lane M, prestained molecular weight markers (Bio-Rad) for SDS–PAGE.

immature VLP (Fig. 7A), and confirmed that unprocessed Gag shells were intact following detergent treatment. The Gag shells were not highly aggregated in our preparations, consistent with studies on avian leukosis virus (Stewart *et al.,* 1990). When the *in vitro* processing products were similarly analyzed, no double ring structures were present but round electron-dense structures, likely corresponding to a complex of NC (possibly including CA-p15) and RNA, were observed (Figs. 7C and 7D). The structures were embedded in, and attached to, partially disrupted spherical shells (Fig. 7C, arrowheads), which may correspond to a multimer of predominantly MA, although this is not proven. A high degree of aggregation between the shells was frequently visible (Fig. 7C, right). Characteristic of most of the cores was the absence of core shells that were normally observed in mature HIV cores (compare Figs. 7D and 7E). The occasional core shell visualized at a low frequency was neither conical nor rod-shaped (Fig. 7C, arrow). No core shells were visible when the processing reaction went to completion, conditions under which the majority of the CA molecules were dissociated to monomers (data not shown). Together, these results suggest that the *in vitro* processing of Gag shells allowed formation of condensed cores and outer shells but did not routinely produce core shells.

DISCUSSION

Despite reports that immature forms of avian retroviral and HIV particles could be cleaved by exogenous PR in

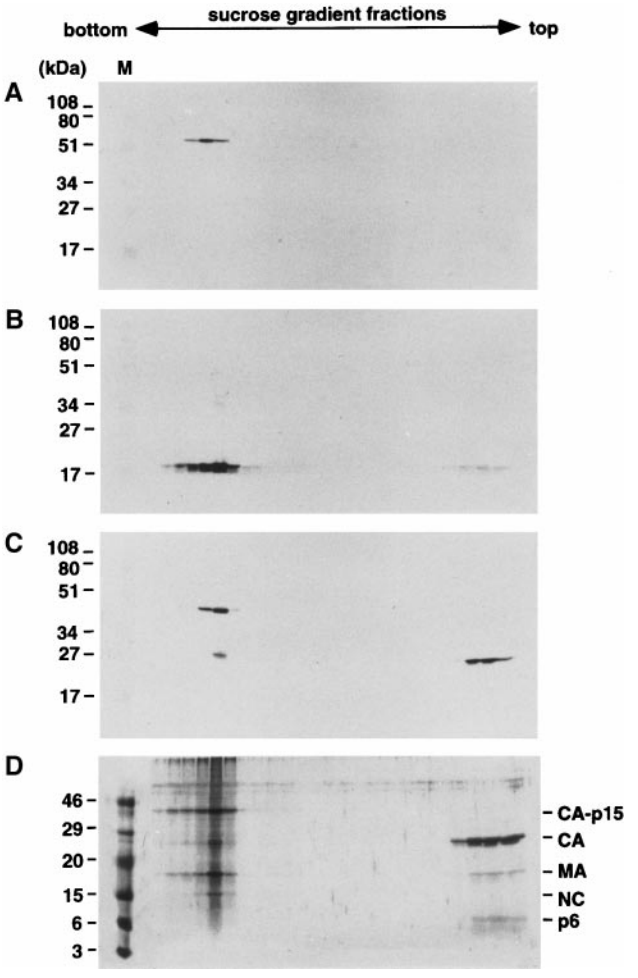

FIG. 5. Gradient analysis of *in vitro* processed Gag shells following delipidization by ether treatment. Immature VLP were extracted with 2 vol of ether for 30 min and pelleted by centrifugation at 4°C at 150,000 *g* for 2 h. Digestion of the delipidized Gag shells was carried out as described in the legend to Fig. 3. The undigested and 30-min digested samples were layered onto 20–70% (w/v) sucrose gradients and centrifuged in an SW55 rotor at 4°C at 120,000 *g* for 4 h. Fractions from the bottom to the top (left to right) were analyzed by SDS–PAGE followed either by Western blotting using anti-HIV-1 MA (B) or CA (A and C) monoclonal antibodies or by silver staining (D). (A) Unprocessed; (B–D) processed. Lane M, prestained molecular weight markers (Bio-Rad in A–C or Life Technologies in D).

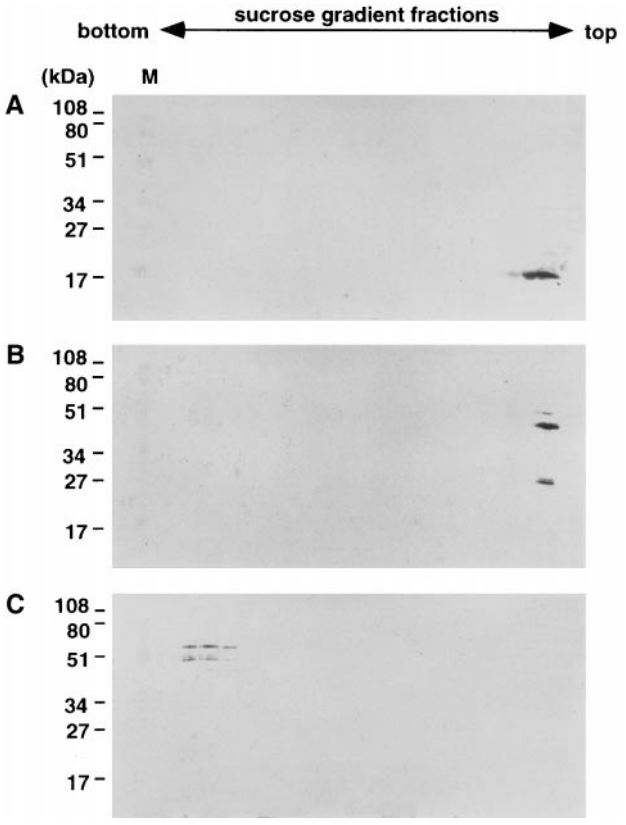

FIG. 6. Gradient analysis of detergent-treated Gag products following *in vitro* processing. Delipidization and digestion of Gag VLP were performed as described in the legend to Figs. 2 and 3, respectively. The 30-min digested products were centrifuged on 20–70% (w/v) sucrose gradients as described in the legend to Fig. 3 and highly multimeric forms of Gags were collected from the relevant gradient fractions. The highly multimeric Gag products were subjected to another treatment with 0.5% Triton X-100 for 30 min and centrifuged on 20–70% (w/v) sucrose gradients in an SW55 rotor at 4°C at 120,000 *g* for 4 h. Fractions from the bottom to the top (left to right) were analyzed by SDS–PAGE followed by Western blotting using anti-HIV-1 MA (A) or CA (B and C) monoclonal antibodies. (A and B) Processed; (C) unprocessed. Lame M, prestained molecular weight markers (Bio-Rad).

the presence of detergent (Stewart *et al.,* 1990; Konvalinka *et al.,* 1995), the morphological analysis of such processed particles has not been previously shown. In this paper we performed *in vitro* processing of immature HIV VLP by exogenous PR following removal of the reagents used for delipidization and observed the structural transformation of the VLP associated with the cleavage reaction. In parallel, we analyzed the multimeric nature of the *in vitro* cleavage products by density gradient analysis. We found that, in the system, the MA/CA junction was cleaved faster than the CA/NC junction, accompanied by a failure to form core shells. By contrast, although incomplete, outer shells and cores still formed.

It is difficult to assess the real order of Gag processing in virus particles budded from infected cells. Indeed, Gag processing is seen even in the cytoplasm (Kaplan and Swanstrom, 1991), although processed Gag proteins may not participate in virus particle formation since it has been reported that premature processing in the cytoplasm by overexpression of PR leads to no virus particle production (Karacostas *et al.,* 1993; Krausslich, 1991; Park and Morrow, 1991). Nevertheless, recent mutational analyses on the cleavage sites suggest an order of Gag processing based on the morphologies of the virus particles arrested at various stages during the maturation process (Accola *et al.,* 1998; Gottlinger *et al.,* 1989; Krausslich *et al.,* 1995; Wiegers *et al.,* 1998). Although an alteration of the Gag conformation by the mutations introduced in those studies cannot be ruled out, the data suggest that cleavage at the p2/NC junction is the first event for NC condensation and is followed by the MA/CA cleavage, leading to formation of a submembrane shell. Formation of a core shell has been suggested to be triggered by cleavage at the MA/CA junction and completed by cleavage at the CA/p2 junction (p2/NC > MA/CA > CA/p2). *In vitro* processing studies in which *in vitro* translated Gag protein was utilized as a substrate also support this order. These studies have shown that Gag protein is initially cleaved at the p2/NC junction, followed by cleavage at the MA/CA junction, and finally cleavage at the CA/p2 junction (p2/NC > NC/p6 ≥ MA/CA > CA/p2) (Pettit *et al.,* 1994; Tritch *et al.,* 1991). However, we observed a different order of Gag processing (MA/CA > p2/NC > CA/p2). Stewart and Vogt (1994) have proposed a structural model for *in vitro* processing of retroviral particles, in which the MA/CA junction may be cleaved initially because PR approaches Gag shells from the outside. Our studies support this view, since our order did not change even when the reaction was carried out at different pH values or salt concentrations, suggesting that alteration of the order in *in vitro* processing is not due to altered binding affinity of PR but rather to physical distances to each of the cleavage sites.

It is interesting that the altered order of Gag processing allowed outer shell formation and core condensation even though their architectures were aberrant. Recent ultrastructural studies of immature HIV particles have suggested that Gag precursors form a fullerene-like particle structure composed of hexamers with pentamers (Nermut *et al.,* 1994) or a cage-like structure (Fuller *et al.,* 1997). Although little is known of how Gag processing leads to collapse of the double ring structure composed of Gag precursors and reassembly to yield the individual Gag architectures of the mature particles, Nermut and Hockley (1996) have suggested that following Gag processing, multimers of CA are dissociated into monomers before reassembly into the conical core shell while, in contrast, those of MA remain associated. We reason that this might lead to a failure of core shell formation but allow outer shell formation in our system although it remains unproven that our observed outer shell was a layer of MA. A failure of core shell formation was also observed when *in vitro* assembled Gag particles were

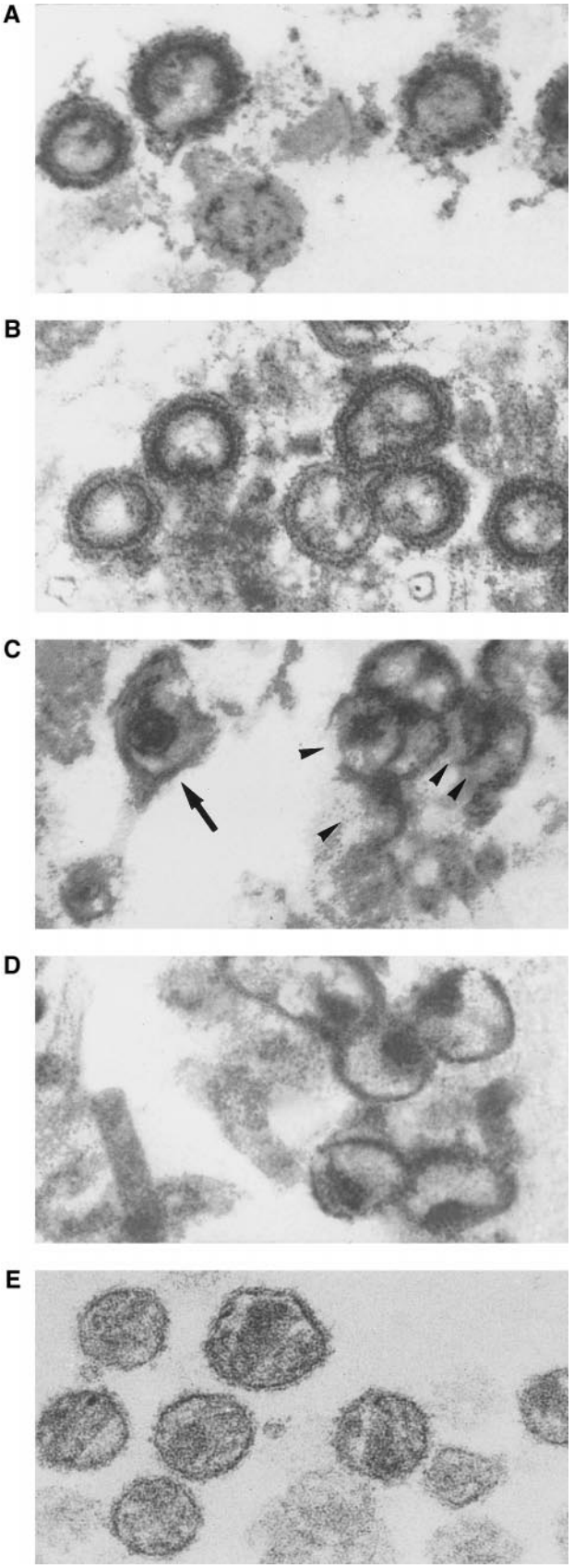

FIG. 7. Electron microscopic examination of unprocessed and *in vitro* processed Gag shells. Delipidization and digestion of Gag VLP were performed as described in the legends to Figs. 2 and 3, respectively. The 30-min digested products were observed by electron microscopy. (A) Immature VLP; (B) delipidized but unprocessed Gag shells; (C and D) delipidized and processed Gag shells; (E) authentic HIV-1 (LAV1 strain) particles. An arrowhead shows a particle containing an partially disrupted outer shell and an electron-dense core. Note the absence of a core shell in most of the *in vitro* digested particles. An arrow shows a particle containing an outer shell and an electron-dense core surrounded by a core shell. All micrographs are at same magnification.

subjected to *in vitro* processing (Gross *et al.,* 1998). Recent crystallographic studies have demonstrated trimerization of MA (Hill *et al.,* 1996; Rao *et al.,* 1995) and, in contrast, dimerization of CA (Berthet-Colominas *et al.,* 1999; Gamble *et al.,* 1996, 1997; Momany *et al.,* 1996; von Schwedler *et al.,* 1998), suggesting that the cleavage at the MA/CA junction plays a key role for morphological conversion of core shells from spheres to cones (Gamble *et al.,* 1997; von Schwedler *et al.,* 1998). It is possible that the *in vitro* processing described here might fail to redirect an assembly state of CA.

MATERIALS AND METHODS

Cells and viruses

For the immature form of HIV VLP, *spodoptera frugiperda* (*Sf*9) cells were inoculated with a recombinant *Autographa californica* nuclear polyhedrosis virus (baculovirus) containing the full-length HIV-1 (IIIB strain) *gag* gene (Morikawa *et al.,* 1996) and cultured at 27°C in TC-100 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS). For the mature form of HIV VLP, RK-13 cells were infected with a recombinant vaccinia virus containing the full-length HIV-1 (IIIB strain) *gag-pol* gene (Hoshikawa *et al.,* 1991) and cultured at 37°C in Eagle's minimum essential medium (Life Technologies) supplemented with 5% FBS.

Purification of HIV VLP

HIV VLP were purified from culture medium of cells infected with recombinant viruses and finally resuspended in PBS. The purification procedure was described elsewhere (Morikawa *et al.,* 1996).

Removal of viral lipid bilayer and *in vitro* processing by HIV PR

To remove the lipid bilayer of HIV VLP, the purified VLP was treated with 0.5% Triton X-100 or 2 vol of ether at room temperature for 30 min. Following dilution with 10 vol of PBS, the denuded Gag shell of immature VLP was pelleted by centrifugation at 4°C at 150,000 *g* for 2 h. For *in vitro* processing, the Gag shell was resuspended with 50 mM MES (pH 6.0), 150 mM NaCl, 1 mM DTT, and 1 mM EDTA unless otherwise indicated. Digestion was carried out with purified HIV-1 PR (Roche Discovery, Welwyn, UK) at 37°C for the indicated time (see legends

to figures) in 100-μl reaction mixtures containing 35–40 μg of Gag and 250 ng of HIV-1 PR (Gag-to-PR molar ratio of 30:1) and stopped by addition of pepstatin A. In some experiments, *in vitro* processed Gag shell was again treated with 0.5% Triton X-100 (see legend to Fig. 6).

Gradient analysis

Unprocessed and processed Gag shells were applied onto 20–70% (w/v) sucrose density gradients in PBS and centrifugal in an SW55 rotor (Beckman) at 4°C at 120,000 *g* for 4 h, conditions under which the Gag shells reached equilibrium density. Alternatively, processed Gag shells were applied onto 15–30% (v/v) glycerol gradients in 20 mM Tris (pH 7.4) and 100 mM NaCl and sedimented in an SW55 rotor at 4°C at 230,000 *g* for 40 h. A low-molecular-weight calibration kit (Amersham Pharmacia Biotech) was used for molecular mass markers in the latter experiment.

Protein detection

Protein samples were analyzed by electrophoresis on SDS–PAGE on 14% acrylamide. Western blotting (Towbin *et al.,* 1979) was carried out using anti-HIV-1 MA or CA monoclonal antibody (Medical Research Council AIDS Reagent Repository, National Institute for Biological Standards and Control, Herz, UK) and anti-mouse IgG alkaline phosphatase conjugate (Cappel). To detect NC and p6, protein samples were analyzed by SDS–PAGE on 16% acrylamide and subjected to silver staining.

Electron microscopy

The procedure for microscopic examination was described previously (Goto *et al.,* 1990). Briefly, HIV VLPs and *in vitro* processed Gag shells were collected by centrifugation and fixed with 2% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) at 4°C for 2 h prior to treatment with 1% osmium tetroxide in 50 mM cacodylate buffer (pH 7.2) at 4°C for 1 h. Ultrathin sections were stained with uranyl acetate and lead citrate. Authentic mature HIV particles were similarly prepared from culture medium of MOLT4/HIV-1 (LAV1 strain) cells.

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